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(54) Title: MONOCLONAL ANTIBODY AGAINST HUMAN RECEPTOR PROTEIN 4-1BB AND METHODS OF ITS USE FOR TREATMENT OF DISEASES

(57) Abstract

Disclosed herein is a monoclonal antibody against H4-1BB that can be used to enhance or suppress T-cell activation and proliferation by treating T-cells that have expressed receptor protein H4-1BB with the anti H4-1BB monoclonal antibody. Also disclosed herein is cDNA for the human receptor H4-1BB. The cDNA of the human receptor H4-1BB is about 65 % homologous to the mouse cDNA 4-1BB and was isolated by using probes derived from murine cDNA 4-1BB. A fusion protein for detecting cell membrane ligands to human receptor protein H4-1BB was developed. It comprises the extracellular portion of the receptor protein H4-1BB and a detection protein (alkaline phosphatase) bound to the portion of the receptor protein H4-1BB. B-cells that have expressed a ligand to receptor protein H4-1BB can be treated with cells that have expressed receptor protein H4-1BB and B-cell proliferation may be induced. The use of H4-1BB to block H4-1BB ligand binding has practical application in the suppression of the immune system during organ transplantation or against autoimmune diseases.

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MONOCLONAL ANTIBODY AGAINST HUMAN RECEPTOR PROTEIN 4-1BB AND METHODS OF ITS USE FOR TREATMENT OF DISEASES.

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of co-pending application Serial No. 08/122,796 filed September 16, 1993, which is a continuation-in-part of co-pending application Serial No. 08/012,269 filed February 1, 1993, which is a continuation-in-part of co-pending application Serial No. 07/922,996 filed July 30, 1992, which is a continuation-in-part of copending application Serial No. 07/267,577 filed November 7, 1988, now abandoned.

The subject matter described herein was in part a subject invention of NIH Grants Nos. IR23AI23058-03, RO1 AI28175 and P60 KD20542 of which the present inventor was the Principal Investigator and either the Donald Guthrie Foundation for Medical Research Inc. of Guthrie Square, Sayre, Pennsylvania 18849-1669 or Indiana University School of Medicine of Indianapolis, Indiana 46202, was the Grantee.

FIELD OF THE PRESENT INVENTION

The present invention relates to a previously unknown human receptor protein, H4-1BB, a monclonal antibody that specifically recognizes H4-1BB and a ligand protein to H4-1BB. H4-1BB was isolated and identified by research on a homologous murine (mouse) receptor protein, 4-1BB, which was isolated and identified by specific expression of the T cell genes by the present inventor.

BACKGROUND OF THE PRESENT INVENTION

The immune system of humans and other species require that white blood cells, which include phagocytes, T lymphocytes and B cells, be made in the bone marrow. As presently understood, the phagocytes include macrophage cells which scavenge unwanted materials such as virus proteins from the system. The lymphocytes include helper T cells and killer T cells and B cells as well as other cells, including those categorized as suppressor T cells. The B cells produce the antibodies. The killer T cells physically pierce the cell and the helper T cells facilitate the whole process. The immune process is facilitated by lymphokines.

Lymphokines are the proteins by which the immune cells communicate with each other. Scientists produce them in sufficient quantities for therapeutic use against immunologic diseases. There are many known lymphokine proteins and they include the interferons, interleukin-1,2,3,4,5,6,7, colony-stimulating factors, lymphotoxin, tumor necrosis factor and erythropoietin, as well as others.

Interleukin 1, secreted from macrophages activate the helper T cells and raise the body temperature causing fever which enhances the activity of the immune cells. The activated helper T Cells produce Interleukin 2, which in turn stimulates the helper and killer T cells to grow and divide. The helper T cells also produce another lymphokine, B cell growth factor (BCGF), which causes B cells to multiply. As the number of B cells increases, the helper T cells produce another lymphokine known as the B cell differentiating factor (BCDF), which instructs some of the B cells to stop replicating and start producing antibodies. T cells also produce a lymphokine, gamma interferon (IF), which has multiple effects like Interleukin 2. Interferon helps activate killer T cells, enabling them to attack the invading organisms. Like BCGF, interferon increases the ability of the B cells to produce antibodies. Interferon also keeps the macrophages at the site of the infection and helps the macrophages digest the cells they have engulfed. Gathering momentum with each kind of lymphokine signal between the macrophages and the T cells, the lymphokines amplify the immune system response and the virus protein or other foreign matter on the infected cells is overwhelmed. There are many other lymphokines, maybe a hundred or more, which participate in the immune process. Many lymphokines are known and many are not.

Lymphokine activities are produced when a certain lymphokine binds to its specific receptor. Among scientists there is widespread use of cloned cell lines for production of lymphokines and their receptors. The isolation of lymphokine and lymphokine receptor mRNA has become a common technique. The mouse receptor protein, 4-1BB, was isolated and identified based on specific expression of the T cell genes using a technique identified by the present inventor in a publication (Proc. Natl. Acad. Sci. USA. 84, 2896-2900, May 1987, Immunology). The protocol reported in this publication can be used by scientists to detect virtually all of the lymphokines. The method is designed to detect virtually all mRNA expressed differentially and the mRNA sequences of the immune cells are expressed differentially (as they relate to the T cells and the killer T cells) even though the level of expression is low and the quantity of the lymphokine and its receptor protein is low. The present inventor believes that the analysis described in the above identified publication can reveal biologically important molecules such as lymphokines and their receptors because there are many indications that biologically important or active molecules are coded by the most scarce messages.

Most T cell factors have been classically identified by recognizing biologic activities in assays, purifying the protein information. An alternative approach is to isolate putative T cell genes based upon specific expression and then demonstrate the function of the unknown molecule. Using the aforesaid modified differential screening procedure, the present inventor cloned a series of T cell subset-specific cDNAs from cloned helper T (HTL) L2 and cloned cytolytic T lymphocyte (CTL) L3.

T cells are critically important in acquired immunity, providing protection against viral, bacterial and parasitic infection. T cells are activated when they encounter peptide from the invading pathogen in context with self-MHC via the T cell's own T cell receptor (TCR) complex and other costimulatory molecule(s), such as CD28(1,2). Without the engagement of the other co-stimulatory molecule(s) the T cell is rendered anergic(3). To date the best characterized co-stimulatory molecule has been CD28(1,2). More recently, however, other cell surface molecules have been suggested to play a co-stimulatory role, such as the molecule 4-1 BB. 4-1 BB is a ~55 kDa homodimeric molecule expressed on activated T cells in the mouse and is a member of the Nerve Growth Factor receptor

(NGFR)/Tumor Necrosis Factor receptor (TNFR) gene family(4). This family is characterized by the presence of cysteine-rich motifs in the extracellular domains. Other members of this family include NGFR, B cell activation molecule CD40, the T cell activation molecule OX-40 in rat and CD27, the two receptors for TNF called TNFR-1 and TNFR-11, the apoptotic inducing molecule called Fas, and CD30 which has been suggested to play a role in the regulation of cellular growth and transformation(4,5).

A series of T-cell subset-specific cDNAs were isolated from cloned murine T-cells by employing a modified differential screening procedure. The nucleotide sequence and expression properties of some of the cDNA species have been reported. One of the genes not previously characterized, that encodes mouse receptor protein 4-1BB, was studied further. These studies have led to the isolation of the human homologue to 4-1BB, H4-1BB.

Specific immune responses are governed by the recognition of antibodies to foreign antigens. Antibodies form a family of structurally related glyco-proteins and confer the protective effect of cell-mediated immunity. Antibodies are produced by B lymphocytes and are bound to the cell membrane, functioning as B cell receptors for antigens. Antibodies are also secreted by B cell progeny which differentiate in response to stimulation by antigens. A specific antigen will trigger the complementary B lymphocyte to proliferate and differentiate into effector cells which then eliminate the antigen. Each lymphocyte produces an antibody of a particular specificity, and thus immune responses are very specific for distinct antigens. The portion of the antigen recognized by T and B lymphocytes are called epitopes or determinants.

The development of techniques to produce virtually unlimited amounts of a single (monoclonal) antibody for a specific antigenic epitope has had an enormous impact on clinical immunology. To produce a monoclonal antibody of known specificity, a mouse can be injected with a particular antigen, such as a receptor protein and the spleen B lymphocytes (that produce the antibody against the protein) can be fused via somatic cell hybridization to a myeloma (lymphocyte tumor) to produce an immortal cell line. This is done because normal B lymphocytes can not grow indefinitely, yet when fused with the myeloma, the resulting hybridoma does produce a virtually endless supply of a specific monoclonal antibody. "Selection" mediums have been developed to ensure that only the fused cells continue to grow. Each hybridoma cell is specific for only one antigenic determinant. If several hybridomas are

produced that secrete antibodies that recognize and bind to the surface of a particular cell, each hybridoma clone will secrete an antibody for only one surface antigenic determinant. To determine which mAbs specifically bind to the protein receptor, the hybridomas can be screened with ELISA (enzyme-linked immunosorbent assay).

Monoclonal antibodies have numerous applications: 1) The hybridoma can produce large quantities of specific antibodies that are normally either unavailable in small quantities or not available at all; 2) the hybridoma can be directed to produce antibodies against a single antigen determinant which, for complex antigens, may be normally very difficult; 3) pure antibodies can be obtained against antigens that cannot be purified; 4) immunodiagnosis of infectious and systemic diseases by detecting specific antigens circulating in tissues or using monoclonal antibodies in immunoassays; 5) characterization of protein receptors and the role they play in the transition from a naive to a memory T cell; and 6) blocking or enhancing immune response.

SUMMARY OF THE PRESENT INVENTION

The present invention includes the human receptor protein H4-1BB and the cDNA gene encoding for human receptor protein H4-1BB. The nucleotide sequence of the isolated cDNA is disclosed herein along with the deduced amino acid sequence. The cDNA gene identified as pH4-1BB was deposited at the Agricultural Research Service Culture Collection and assigned the accession number: NRRL B21131

The cDNA, including its fragments and derivatives, can be used as a probe to isolate DNA sequences encoding for proteins similar to the receptor protein encoded by the cDNA. The cDNA of the human receptor, H4-1BB, is about 65% homologous to the mouse cDNA 4-1BB and was isolated by using probes derived from cDNA 4-1BB. The cDNA gene identified as p4-1BB was deposited at the American Type Culture Collection at 12301 Parklawn Drive, Rockville, Maryland 20852 under ATCC No.: 67825.

The human receptor protein H4-1BB can be produced by: 1) inserting the cDNA of H4-1BB into an appropriate expression vector, 2) transfecting the expression vector into an

appropriate transfection host, c) growing the transfected hosts in appropriate culture media and d) purifying the receptor protein from the culture media. The protein and fragments and derivatives can be used: 1) as a probe to isolate ligands to human receptor protein H4-1BB, 2) to stimulate proliferation of B-cells expressing H4-1BB ligands, or 3) to block H4-1BB ligand binding.

B-cell proliferation can be induced by treating B-cells that have expressed a ligand to receptor protein H4-1BB with cells that have expressed receptor protein H4-1BB. The use of H4-1BB protein, H4-1BB ligand protein, or fragments of the proteins, to block H4-1BB ligand binding has practical application in the suppression of the immune system during organ transplantation. A similar costimulatory immune system pathway is being analyzed for this type of application. See "Mounting a Targeted Strike on Unwanted Immune Responses", Jon Cohen, Science, Vol. 257, 8-7-92; "Long Term Survival of Xenogeneic Pancreatic Islet Grafts Induced by CTLA4Ig", Lenschow et al, Science Vol. 257, 7-8-92; and "Immunosuppresion in Vivo by a Soluble Form of the CTLA-4 T Cell Activation Molecule", Linsley et al, Science Vol. 257 7-8-92.

A monoclonal antibody against H4-1BB can be used to enhance T-cell proliferation by treating T-cells that have expressed receptor protein H4-1BB with the anti H4-1BB monoclonal antibody. Some tumors are potentially immunogenic but do not stimulate an effective anti-immune response in vivo. Tumors may be capable of delivering antigen-specific signals to T cells, but may not deliver the co-stimulatory signals necessary for full activation of T cells. Expression of the co-stimulatory ligand B7 on of melanoma cells was found to induce the rejection of a murine melanoma in vivo. ("Tumor Rejection After Direct Co-Stimulation of $CD8^+$ T Cells by B7-Transfected Melanoma Cells", Sarah E. Townsend and James P. Allison, Science Vol. 259, 1-5-93.) A monoclonal antibody against H4-1BB may be capable of the same effect as it is now known to enduce T cell proliferation and activation.

The present invention includes a monoclonal antibody 4B4-1 that specifically recognizes an epitope on the extracellular domain of human receptor protein 4-1BB on peripheral blood T cells. The monoclonal antibody is produced from a hybridoma identified as 4B4-1 and deposited under the Budapest Treaty at the American Type Culture Collection at 12301 Parklawn Drive, Rockville, Maryland 20852 under ATCC No.: HB-11860.

The 4B4-1 mAb will have great utility in assessing the role of the 4-1BB receptor protein in the transition from naive to memory T-cells. In addition, by treating T-cells that have expressed receptor protein 4-1BB with 4B4-1 mAb T-cell proliferation and activation can be enhanced. Cross-linking of the 4-1BB with 4B4-1 mAb will produce the effects similar to the binding of the 4-1BB ligand to 4-1BB. The cross-liking effect makes the 4B4-1 mAb very useful for culturing T-cells because cross-linking is necesarry for adequate proliferation. Therefore, the 4B4-1 mAb is a useful adjunct to T-cell culture medium. The mAb can also be used to enhance T-cell activation which is useful in the treatment of cancers that avoid normal immune responses.

A mAb aganist H4-1BB can also be used to interfere with H4-1BB and H4-1BB ligand binding. By interfering with the ligand binding, the immune responses will be suppressed. The mAb is usefull as a tool to elucidate the mechanisms of auto-immune disease and can be used to control such problems. For example, the mAb described herein can be used to rheumatoid arthritis, systemic lupus erythematosis, and diabetis. The blocking effect can also be used to suppress the immune system during organ transplantation.

The monoclonal antibody against the murine 4-1BB ligand was produced by injecting Sprague-Dawley rats with purified recombinant protein of the 4-1BB ligand. The spleen cells were then fused to myeloma cell line SP/O. The positive clones were tested with H4-1BB expressing T-cell line. Each step pf these processes summarized here will be described in detail below.

The monoclonal antibody can be used enhance T-cell proliferation and activation. This can be done by treating T-cells that have expressed receptor protein 4-1BB with anti H41-BB monoclonal antibody.

A fusion protein can detect cell membrane ligands to human receptor protein, H4-1BB. A fusion protein of the present invention comprises the extracellular portion of the receptor protein H4-1BB and a detection protein (alkaline phosphatase) bound to the portion of the receptor protein H4-1BB. The portion of the receptor protein H4-1BB binds to the cell membrane ligands and binding can be detected by relative activity assays for the detection protein. The fusion protein is placed in the presence of a cell suspected to express the receptor

protein H4-1BB. Then the cell is washed of any fusion protein not bound to the cell membrane ligands. Once the washed cells are placed in the presence of a substrate for the detection protein and the relative activity of the detection protein can be measured.

The primary object of the present invention is the identification of the new human receptor, H4-1BB as identified herein by its sequence.

Another object of the present invention is to teach a fusion protein comprising the extracellular portion of H4-1BB and a detection protein.

Still another object of the present invention is to teach methods of using the cDNA H4-1BB, the receptor protein H4-1BB, the monoclonal antibody and the ligand for H4-1BB.

BRIEF DESCRIPTIONS OF THE FIGURES

Figures 1a and 2b illustrate the molecules involved in T-cell activation.

Figures 2a, 2b, and 2c illustrate a normal T-cell activation pathway.

Figures 3a, 3b, and 3c illustrate CTLA4-Ig alone, 4-1BB/AP and CTLA4-Ig together and 4-1BB/AP alone respectively being used to block steps in the T-cell activation pathway.

DETAILED DESCRIPTION

In the following detailed description references are made to known procedures and studies, as well as published work of the applicant. These publications are incorported herein by reference for clarity and listed in an Appendix A at the end of this detailed description. A list of common abbreviations is included in Appendix B.

The following description teaches the isolation of 4-1BB and its human homologue, H4-1BB, the preparation of the peripheral blood cells, including the antibodies and reagents used, the production of fusion protein, immunization and monoclonal antibody production, and immunoprecipitation studies. In short, the research shows that the

monoclonal antibody 4B4-1 specifically binds to human receptor protein 4-1BB on peripheral blood T cells. The results are coupled with the precise detail of the methods employed.

Isolation and characterization of mouse receptor 4-1BB

SEQ ID NO:1 shows the nucleotide sequence and the deduced amino acid sequence of the mouse receptor 4-1BB. The predicted amino acid sequence is shown below the nucleotide sequence. The transcript of 4-1BB was inducible by concanavalin A in mouse splenocytes, T cell clones, and hybridomas. The expression of 4-1BB transcripts was inhibited by cyclosporin A. The 4-1BB mRNA was inducible by antigen receptor stimulation but was not inducible by Il-2 stimulation in the cloned T-cells (1). The 4-1BB cDNA encodes a peptide of 256 amino acids containing a putative leader sequence, a potential membrane anchor segment, and other features of known receptor proteins. Therefore, the expression pattern of 4-1BB resembles those of lymphokine mRNAs while the sequence appeared consistent with those of receptor proteins.

The major species of 4-1BB on the cell surface appears to be a 55-kDa dimer. 4-1BB also appears to exist as a 30-kDa monomer and possibly as a 110-kDa tetramer. Since these 4-1BB species were immunoprecipitated from a homogeneous population of cells (T-cell clone f1), all forms potentially co-exist on each cell. We will need to compare the peptide digests from the 4-1BB monomer and dimer to determine whether 4-1BB exists as a homodimer on the cell surface. A variety of cell surface receptors such as the insulin receptor(2), the B cell surface immunoglobin receptor (3), the T cell Ag receptor (4), the CD28 costimulatory receptor (5), and the CD27 T-cell antigen (6) are composed of disulfide-bonded subunits. Receptor dimerization may be required for ligand binding and subsequent biochemical signalling.

4-1BB is not expressed on resting T cells but is inducible by activators which deliver a complete growth stimulus to the T cell. The combination of PMA and ionomycin is capable of mimicing those signals required for T cell proliferation. Although PMA or ionomycin alone induced 4-1BB mRNA, the combination of PMA and ionomycin resulted in optimal 4-1BB

expression. Furthermore, the expression of 4-1BB was not transient. When purified splenic T-cells were stimulated with immobilized anti-CD3, 4-1BB mRNA was expressed and this expression was maintained for up to 96 hrs poststimulation. Cell cycle analysis will be required to confirm that 4-1BB is expressed throughout cell cycle progression.

4-1BB is structurally related to members of the nerve growth factor receptor super-family. Although these receptors possess structurally similar ligand-binding properties (cysteine-rich regions), the cytoplasmic domains of these proteins are nonconserved which could allow for diversity in transmembrane signaling. Some members of this family are involved in the T or B cell activation process. There are in vitro functional data on the OX-40, CD4O and CD27 antigens. Antibodies against the OX-40 augment the T-cell response in a mixed lymphocyte reaction (7) and antibodies against CD4O enhance B-cell proliferation in the presence of a coactivator, such as PMA or CD2O antibodies, and synergize with IL-4 in vitro to induce B-cell differentiation and to generate long-term normal B cell lines (8). One monoclonal antibody, anti-1A4, which recognizes an epitope on the CD27 molecule inhibited calcium mobilization, IL-2 secretion, helper T cell function, and T-cell proliferation. On the other hand, CLB-CD27/1, another anti-CD27 mAb enhanced proliferation of human T cells stimulated with PHA or anti-CD3 mAb (6). These results indicate that the CD27 molecule plays an important role in T cell activation. Except for TNFRs, NCFR and CD4O, the ligands or cell surface molecules to which the members of the superfamily bind are not yet identified. Identification and characterization of the ligands to which the receptors bind will be helpful in better defining the physiologic role of 4-1BB.

To ascertain whether cell surface 4-1BB could contribute to T cell activation, the anti-4-1BB 53A2 was used as an antagonist to 4-1BB. These data suggested that 4-1BB does in fact have the potential to function as an accessory signaling molecule during T cell activation and proliferation. The addition of soluble 53A2 to purified splenic T cells stimulated with immobilized anti-CD3 resulted in an amplification of ^{3}H thymidine incorporation compared to T cells stimulated with anti-CD3 alone. This pattern of enhancement ranged from 2- to 10-fold in three independent experiments.

In the original two signal model of Bretcher and Cohn, they proposed that signal 1, the occupancy of the T cell antigen receptor (TCR), resulted in inactivation of the T cell in the

absence of signal 2, which is provided by accessory cells. This has since been confirmed by a variety of studies (9). The identification of the accessory cell CD28 as a potent costimulatory receptor on T cells was a significant contribution in beginning to charactize the accessory signal(s) required for optimal T cell proliferation (10). It is possible that other cell surface molecules may contribute to these costimulatory activation requirements (11).

The biochemical signals delivered through 4-1BB are not completely known. One possibility considered was the observation that 4-1BB contains a putative $p56^{lck}$ tyrosine kinase binding domain in its cytoplasmic tail. It was later determined that $p56^{lck}$ tyrosinase kinase binds to 4-1BB. It will also be worthwhile to determine if 4-1BB-mediated signaling can regulate genes such as IL-2 and IL-2 receptor, whose expression is required for T cell activation and subsequent proliferation.

The precise functions of members of the Nerve Growth Factor Receptor (NGFR) family appear to be diverse. An emerging theme of inquiry concerns the ability of these molecules to maintain the responsiveness or viability of the particular cell type in which they are expressed. For instance, NGF is absolutely required for viability of neurons *in vitro* and *in vivo* (12). The crosslinking of CD4O by soluble antiCD40 monoclonal antibody blocks germinal center centrocytes from undergoing apoptosis in vitro (13). Signals delivered through CD4O may also aid in maintenance of responsiveness to differentiation factors. The ligation of CD4O with anti-CD4O $F(ab')_2$ fragments in the presence of IL-4 induced large increases IgE synthesis (14). Also, anti-CD40 activated naive B cells treated with IL-10 and transforming growth factor-ß became committed to IgA secretion (15). In addition to sharing the molecular characteristics with the NGFR superfamily, it was noted that the 4-1BB contained a putative zinc finger structure of the yeast elF-2b protein (16). 4-1BB also shares a conserved region with the *sina seven in absentia of Drosophila*, which is required for correct photoreceptor cell development (17). That particular region is also similar to the protein product of the DGI7 gene of *Dictyostelium*, whose expression is specificaily induced during aggregation by cAMP (18).

This region forms the pattern of $C\text{-}X_2\text{-}C\text{-}X_9\text{-}C\text{-}X_3\text{-}H\text{-}X_3\text{-}C\text{-}X\text{-}C$; and the cysteines and histidine are conserved in a similar space in 4-1BB, *sina*, and DGI7 proteins. Ten of 24 amino acids between the 4-1BB and *sina* proteins are identical; 3 of 24 are conservative substitutes. The conserved pattern suggests that these amino acids are functionally important. The *sina*

protein is localized in the nucleus, suggesting that it has a regulatory function in cells. The fact that the amino acid sequence of 4-1BB contains features like a zinc finger motif, a nuclear protein, and a receptor domain suggests that 4-1BB may play diverse roles during cellular proliferation and differentiation.

In addition, 4-1BB may represent another cell-surface molecule involved in T cell-APC interactions. The 4-1BB-AP fusion protein specifically bound to mature B-cell lines, anti-μ-activated primary B cells, and mature macrophage-cell lines. 4-1BB-AP bound at low or insignificant levels to immature B- and macrophage-cell lines, T cell clones, T cell lines, primary culture T cells, and various nonlymphoid-cell lines. Since 4-1BB-AP binds to mature B cells and macrophages, it is possible that signals delivered upon 4-1BB binding may modulate APC functions in some way. This possibility remains to be explored.

Chalupny and colleagues (19) have proposed that 4-1BB Rg, a fusion protein consisting of the extracellular domain of 4-1BB and the Fc region of human IgG, bound to the extracellular matrix (ECM). The highest level of 4-1BB Rg binding was to human vitronectin. In data not shown, an ELISA was performed using 4-1BB-AP and human vitronectin (Yelios Pharmaceuticals/GIBCO-BRL, Grand Island, NY) immobilized at 0.007 mg-10mg per well on microtiter plates. No binding of 4-1BB-AP based on AP activity was observed. To rule out the possibility that 4-1BB-AP was binding to proteins extrinsically attached to the cell surface (possible extracellular matrix components), B-cell lymphomas were washed in acid conditions prior to the binding assay. 4-1BB-AP still bound specifically to mature B-cell lymphomas. It is still to be determined whether a 4-1BB-ligand specifically expressed on B cells and macrophages exists, and whether 4-1BB-AP may bind to the ECM under particular binding conditions. It is possible that the ECM could facilitate the binding of 4-1BB to a specific cell-surface ligand.

B cells and helper T cells interact with each other through receptors on B cells binding to their specific counter-receptors on T cells. Researchers believe that this interaction results in a cascade of biochemical signaling relays between these two cell types (20). As this interaction proceeds, these cells become committed to enter the S phase of the cell cycle. Initial interactions between TCR and CD4 on T cells, and processed antigen-MHC II on B cells, do not result in B cells capable of entering the cell cycle (21). However, studies from in vitro

systems suggest that once T cells are stimulated, they express newly synthesized or modified cell-surface molecules capable of inducing B cells to enter the cell cycle (22, 23). This T cell function is not antigen-specific or MHC-restricted (24). In addition, soluble factors are not required for the activated Th induction of B-cell activation (25). Once B cells enter the cell cycle, IL-4 induces B cells to progress from G_1 to S phase. The ability of activated T cells or T-cell membranes to promote the entry of B cells into the cell cycle can be blocked by either cycloheximide or cyclosporin A treatment (26, 27). These newly expressed membrane proteins appear to be "lymphokine-like" in their induction characteristics.

4-1BB has expression properties which meet the requirements of a B-cell costimulator. 4-1BB is inducible by anti-CD3 or TCR-mediated T-cell stimulation, and its expression is sensitive to cyclosporin A as well as cycloheximide treatment (28). Interestingly, paraformaldehyde-fixed SF21-4-1BB cells, synergized anti-μ and induced B-cell proliferation. The costimulation of splenic B cells by SF21-4-1BB occurred at optimal (10 μg/ml) and suboptimal (1.0-0.1 mg/ml) doses of anti-μ. The addition of SF21-4-1BB cells to resting B cells, did not result in significant B-cell proliferation. SF21-4-1BB cells did not synergize with TPA or ionomycin, or suboptimal concentrations of LPS in inducing B-cell proliferation.

Although the baculovirus system has been used to express large amounts of recombinant soluble proteins, this system may be utilized for the expression of recombinant cell-surface proteins. The baculovirus infection provides a convenient means to express uniformity high levels of recombinant protein on a per cell basis. It is noteworthy, that the addition of SF21 cells alone did not result in significant levels of costimulation. This can be a potential problem when using cos- or L- cell lines which can exhibit strong costimulator activity on their own.

Another member of the NGFR superfamily, CD40, is expressed on B cells and interacts with gp39, a molecule expressed on activated T cells. The cDNAs encoding the murine (29) and human (30) gp39 proteins have been cloned; this cell surface molecule is a type II membrane protein with homology to tumor necrosis factor. Noelle et al. (31) found that a CD40-immunoglobulin fusion protein, is capable of blocking T cell-induced B-cell proliferation and differentiation in a dose-dependent manner. Armitage et al. have isolated a cDNA for murine gp39 and showed that gp39 could induce B-cell proliferation in the absence

of co-stimuli, and result in IgE production in the presence of IL-4-. Hollenbaugh et al. (32) have shown that COS cells transfected with human gp 39 can synergize with either TPA or anti-CD2O in inducing human B-cell proliferation and is able to stimulate B cells without a costimulator only at low levels. These data indicate that CD4O may be one of the B-cell-surface molecules that transmit signals during physical contact with T cells.

Cell-surface receptors communicate with their external milieu by interacting either with soluble factors or other cell surface molecules expressed on neighboring cells. The role of biochemical signals delivered by cell-cell contact versus those delivered by soluble factors interacting with cell surface receptors is not clear. The NGFR superfamily is unusual for the TNFR I and II as well as the NGFR bind to more than one ligand. The TNFRs I and II both bind to TNF-a and TNF-R (33). The NGFR binds to NGF, brain-derived neurotrophic factor, and neurotrophin-3 (34).

In addition, one ligand may function as both a cell surface and soluble ligand. Recent evidence on the CD40 ligand, gp39, suggests that this ligand can exist as a membrane bound as well as a soluble ligand (35). It may be possible that 4-1BB is secreted and interacts with B cells in a soluble form as well as a membrane bound form. A member of the NGFR receptor family, CD27, which is expressed on T cells, is secreted in addition to being expressed on the cell surface (36). It is also possible that more than one 1 ligand (soluble and cell surface) may bind to 4-1BB.

Isolation of the human homologue, H4-1BB

In order to isolate the human homologue (H4-1BB) of mouse 4-1BB two sets of polymerase chain reaction (PCR) primers were designed. To design the PCR primers, the amino acid sequence among the members of nerve growth factor receptor (NGFR) superfamily were compared because 4-1BB is a member of the superfamily (37). The amino acid sequences employed were mouse 4-1BB (38), human NGFR (39), human tumor necrosis factor receptors (33), human CD40 (40), and human CD27 (6). The areas of sequence conservation among the NGFR superfamily were chosen.

(NOTE: WILL BE REVISED TO REFER TO SEQUENCE LISTINGS - STILL BEING PREPARED)

Forward primer I (H4-1BBFI) spans from amino acids 36 to 41 and forward primer II (HR-1BBFII) spans from amino acids 52 to 58 of the mouse 4-1BB. Reverse primer I (H4-1BBRI) spans from amino acids 116 to 121 and reverse primer II (H4-1BBRII) spans from amino acids 122 to 128 of mouse 4-1BB. The regions used as PCR primers in mouse 4-1BB are indicated if Fig. 1.

The degenerative oligonucleotide sequence of each primer are shown in the Sequence Listing as follows:

SEQ ID NO:3	H4-1BBFI
SEQ ID NO:4	H4-1BBFII
SEQ ID NO:5	H4-1BBRI
SEQ ID NO:6	H4-1BBRII

Peripheral blood lymphocytes from normal healthy individuals were isolated and activated with PMA (10 ng/ml) and ionomycin (1 mM). mRNA from the lymphocytes was isolated. Using reverse transcriptase the human lymphocyte mRNA was converted to single-stranded cDNA. The cDNA was then amplified with Taq polymerase with combination of the primers. The combination of primers was as follows: H4-1BBFI vs H4-1BBRI; H4-1BBFI vs H4-1BBRII; H4-1BBFII vs H4-1BBRI; and H4-1BBFII vs H4-1BBRII.

The primer set of H4-1BBFII and H4-1BBRII produced a specific band of ~240bp. The 240bp is an expected size of human 4-1BB if the human homologue protein is similar to mouse 4-1BB in size. The PCR product (240bp) was cloned in PGEM3 vector and sequenced. One open reading frame of the PCR product was ~65% identical to mouse 4-1BB. Therefore, it was concluded that the 240 bp PCR product is the human homologue of mouse 4-1BB. The 240 bp PCR product was used to screen λgt11 cDNA library of activated human T lymphocytes. An ~0.85 kb cDNA was isolated. The sequence of the cDNA is shown in SEQ ID NO:7 and the predicted amino acid sequence is shown in SEQ ID NO:8

An expression plasmid to produce H4-1BB-AP fusion protein was constructed.

The 5' portion of the H4-1BB cDNA including sequences encoding the signal sequence and the entire extracellular domain, was amplified by PCR. For correctly oriented cloning, a Hind III site on the 5' end of the forward primer and a Bg1 II site on the 5' end of the reverse primer were created.

The Hind III - Bg1 II H4-1BB fragment was inserted into the mammalian expression vector APtaq-1, upstream of the coding sequence for human placental alkaline phosphatase (AP). The oligonucleotides PCR primers used for the amplification of 5' portion of H4-1BB are shown in the Sequence Listing as SEQ ID NO:9 for the forward primer and SEQ ID NO:10 for the reverse primer.

H4-1BB-AP will be used to identify cells and tissues that express ligand for human 4-1BB (i.e. H4-1BBL). The studies with mouse 4-1BB indicated that the ligand for 4-1BB is on the cell suface. B cells and macrophages were major cells that express 4-1BBL. It is expected that H4-1BBL also expresses on human B cells and macrophages.

A mammalian expression cDNA library will be generated from human cell lines that express H4-1BBL. The library will be screened by [^{125}] I-labeled H4-1BB-AP. cDNA for H4-1BBL will then be isolated and characterized. Soluble recombinant H4-1BBL will then be produced. Both H4-1BB-AP and H4-1BBL will be used to suppress or enhance immune responses as described below. Monoclonal antibody to H4-1BBL will be produced and monoclonal antibody to H4-1BB is discussed below.

According to studies with murine 4-1BB, 4-1BB acts as a costimulatory signal. It is expected that H4-1BB will act as a costimulatory signal for T cell activation. Mouse 4-1BB helped B cells with proliferation and differentiation. It is expected that H4-1BB will do the same. H4-1BB-AP, H4-1BBL and monoclonal antibody can be used to suppress or enhance human immune responses.

Figures 1a and 1b illustrate the molecules involved in T-cell activation. During early T-cell activation (cognitive phase), resting T cells express the TCR/CD3 complex and other "accessory" molecules. Among these constitutively expressed molecules, CD4 (or CD8),

LFA-1 and CD28 are probably the ones to receive costimulatory signals. Initial interaction with the TCR/CD3 complex in combination with these 'accessory' costimulatory signals leads to subsequent expression of additional receptor molecules such as CD28, CTLA4, and 4-1BB. These newly expressed molecules are probably going to receive additional important costimulatory signals at later stages of T-cell activation (clonal expansion).

Suppression of immune responses.

Figures 2a-c illustrate a normal T-cell activation pathway. Figures 3a-c illustrate the blocking of immune responses with soluble chimera of 4-1BB. If 4-1BB plays a role in T-cell activation, blocking of the interaction to its ligand on antigen-presenting cells should result in suppression of T-cell dependent immune responses. It is well documented that blocking of the interaction of CD28 to its counter-receptor B7 suppresses in varying degrees, both in vivo antibody production and cell-mediated immune responses. Blocking of both interactions should result in a more effective immunosuppression; since 4-1BB is induced during T-cell activation. Blocking of the interaction of 4-1BB to its ligand may be of importance at later stages of the activation process where the CD28/B7 interaction may no longer be of relevance.

As illustrated with mouse receptor 4-1BB and mouse ligand 4-1BBL above, addition of H4-1BB-AP will coat the H4-1BBL expressing cells and block the normal interaction between H4-1BB and H4-1BBL. This will lead to immunosuppression. This type of immunosuppression is antigen-specific. Therefore it avoids the generalized immunosuppression produced by antiCD3 or cyclosporin A treatments. H4-1BB-AP treatment can be used to treat certain autoimmune diseases and to facilitate organ transplantation.

Immune enhancement.

H4-1BB may function at the late stage of T cell activation and may be a critical molecule for completion of T cell activation. Most tumors display tumor-specific antigens. One reason, however, why immunogenic tumors can escape host immunity is that tumor-reactive T cells receive inadequate costimulation. The introduction of the costimulatory

molecules, such as H4-1BB into the tumor, therefore, could enhance the antitumor immunity of cytotoxic T cells (CTL). H4-1BBL can be expressed in cell-specific fashion. For example, the H4-1BBL can be expressed in melanoma using melanocyte-specific promoter such as tyrosinase promoters. The H4-1BBL-expressing melanoma will stimulate cytotoxic T cells through H4-1BB and activate the melanoma-specific CTL. The activated melanoma-specific CTL can destroy melanoma.

Monoclonal Antibody to H4-BB

4-1 BB is expressed on activated but not resting murine T cells. Crosslinking of 1 AH2 mAb directed against murine 4-1 BB has been shown to enhance anti-CD3-induced T cell proliferation (41). Normal splenic cell antigen presentation and T cell activation can be blocked by inhibiting the binding of 4-1BB on T cells to its ligand on B cells and macrophages with 4-1 BB/AP, a fusion protein containing the extracellular domains of 4-1BB and alkaline phosphatase. We developed and here describe the characterization of human 4-1BB specific mAbs: 4B4-1, 4B11 and 1G5. All mAb specifically bind human 4-1BB expressing SF-21 cells.

Production of recombinant human 4-1 BB

PGEX-3 expression vector (Pharmacia) containing the full length cDNA sequence encoding 4-1 BB and the GST-binding domain of glutathionine S transferase (GST) was constructed and the fusion protein expressed in bacteria. Fusing H4-1BB with GST, allowed for efficient purification of rH4-1BB when isolated by GST-sepharose and a Sepharose 4B column chromatographies. The GST-binding domain was cleaved prior to immunization. The rh4-1BB fraction was purified by GST-sepharose column and Sepharose 4B column chromatographies and subsequently cleaved with factor Xa to release the h4-1 BB portion prior to immunization.

BALB/c animals were immunized with rh4-1 BB protein and their splenocytes were fused with the Sp2/o fusion partner. Eight week-old BALB/c mice were immunized with

50 μg of sh4-1 BB emulsified in Titermax (Cytkx) or Complete Friends adjuvant. Three intraperitoneal (ip) injections were administered 2-weeks apart (42). Three days following the last injection, the mouse was sacrificed and their spleens were removed. Spleen cells were fused with Sp2/o myeloma cells. Spleen cells and Sp2/o cells were mixed at 5:1 ratio and fused using 50% PEG. Cells were washed, resuspended in OptiMEM (Gibco), 10% FCS, 5 mM hypoxanthine, 1 % aminopterin, and 0.8 mM thymidine (HAT) and cultured in 96 well U-bottom plates (Corning). Resulting cell supernatants were screened by ELISA for rh4-1 BB reactivity. Clones were isolated and subcloned.

Antibodies and reagents used

FITC-labeled goat anti-mouse mAb with specificity for the heavy chain of IgG and IgM (GAMGM)(Jackson Laboratories, MA) was used at 1:150. Tricolorlabeled anti-CD4, CD8, CD45RO, PE-labeled anti-CD3 mabs and isotype controls were purchased from CALTAG (S. San Francisco, CA) and used according to manufacturer's recommendations. 4B4-1 (IgGl) was purified over a protein-G sepharose,column and directly labeled by incubating the purified mAb in 0.4 mg/ml of FITC (Pierce) on ice for 3 hrs followed by filtration through Centricon-30 Microconcentrators (Amicon) to remove free-unbound FITC. FITC-labeled 4B4-1 was used at 3-4 μg per 5.0 x 10^5 cells for staining.

The resulting hybridomas supernatants were screened for binding to rh4-1 BB protein by ELISA. Cell supernatants scoring positive for binding to rh4-1 BB by ELISA were subsequently assessed for staining of CEM (human T lymphoma) cells by FCM. CEM and 4B4-1, IG5, and 4B11 hybridoma cells were maintained in the basic culturing media. CEM cells were stimulated overnight to 48 hrs with 10 ng/ml PMA and 1 μM ionomycin in culturing media. Only 4B4-1 mAb specifically bound CEM cells above the non-specific binding of the isotype control and only after the cells had been stimulated with 10 ng/ml PMA and 1 μM ionomycin for 24 hrs. All other mAb positive by ELISA for rh4-1 BB did not appear to bind CEM cells.

To conduct the immunoprecipitations, cell surface proteins were labeled with biotin as previously described (17). Briefly, 1 x 10^7 cells were washed in ice-cold PBS,

resuspended in freshly prepared 0.5 mg/ml animohexanoyl-biotin-N-hydroxysuccinimide ester (AH-BNHS, Zymed Lab., Inc) in PBS for 30 min at 4 C with constant agitation. Cells were washed twice with 0.2 M glycine in PBS. Cells were lysed on ice for 15 min in 200 µl lysis buffer containing 1.0% NP-40, 20 mM Tris-HCl, 0.15 NaCl, µmM sodium orthovanadate, 5 µl/ml aprotinin and 1 µg/ml leupeptin, pH 7.5. Suspension was centrifuged at 14,000 rpm for 10 min, 4 C to remove debris. 20 µl of hybridoma culture supernatants were added for 1 hr, 4 C followed by the addition of 30 µl of a 50% suspension of protein G-Sepharose 4B (Zymed Lab, Inc.) and incubated for 1 hr, 4 C. Suspension was centrifuge and resulting protein G-Sepharose pellet was washed. The immunoprecipitates were boiled 5 min in 20 µl SDS sample buffer and run on 10% SDS-PAGE. Proteins were transfered to Immobilon-P membrane (Millipore Corp., Bedford, MA) and blocked with 5% BSA in buffer containing 25 mM Tris-HCl, 0. 15 NaCl, 0.05% Tween 20 , pH 7.5 (TBST). The blot was probed with avidin-horse radish peroxidase conjugate and developed using chemiluminescence detection kit (ECL, Amersham Corp., Arlington Heights, IL).

To conduct the Flow Cytomertric studies (FCM) fresh cells were washed once and cultured cells were washed 3 times in staining media consisting of PBS, 1 % BSA and 0. 1 % glucose prior to staining. Approximately 2.5-5 x 10^5 cells were resuspended in 50-100 µl of diluted mAb in staining media and incubated at 4 C for 30-60 min. Cells were washed once, resuspended in a 1:150 dilution of FITC-labeled GAMGM secondary mAb (Jackson Laboratories, MA) when needed, incubated 30 min and washed 3-4 times or simply washed 3-4 times if primary mAb were directly labeled. Cells were fixed with 1% paraformaidehyde for overnight prior to analysis on the FACScan by Becton Dickinson using Consort 30 or FACScan software. Appropriate isotype controls were included for all primary antibodies used. Gates were set on live cells only, based on forward verses side scatter profiles. Five to 15000 events were collected for each sample.

To further address whether 4B4-1 mAb specifically recognized 4-1 BB and to assess the specificity of the other mAb picked-up by ELISA, SF-21 insect cells were infected with baclovirus containing cDNA encoding h4-1 BB or an irrelevant protein.

SF-21 cells were grown in Grace Media (Gibco) supplemented with 10% FCS, 3.3 g/i yeastolate (Gibco), 3.3g/l lactoalbumin hydrolysate(Gibco), 2 mM glutamine (Sigma) and antibiotics at 27 C.

Infected SF-21 insect cells were then analyzed by FCM for binding of the various mAbs. Only cells infected with the baclovirus containing and expressing products the h4-1 BB construct on the cell surface, but not the construct encoding and expressing an irrelevant protein were found to bind 4B4-1, 4Bl1 and lG5 mAbs. This data demonstrates that 4B4-1 mAb is specific for h4-1 BB. By immunoprecipitation, only 4B4-1 mAb precipitated a ~35 kDa protein when run on SDS-PAGE under reducing conditions and ~70 kDa protein under non-reducing conditions from rh4-1 BB expressing SF-21 cells, but not those cells expressing irrelevant protein. Similar results were seen using CEM cells with PMA and ionomycin stimulation. IG5 is of an IgM isotype, thus explaining its apparent inability to immunoprecipitate rh4-1 BB. 4B4-1 and 4Bl1 mAb are IgGls.

To evaluate 4-lBB expression in human peripheral blood, mononuclear cells were isolated by passage over ficoll, washed and stained for with 4B4-1 mAb. Human peripheral blood mononuclear cells (PBMC) were isolated by Histopaque 1077 (Sigma) for 30 min at 400 g and the resulting interface cells were washed twice prior to use. Human T cells were isolated according to manufacturer's instructions. Briefly, PBMCs were treated with Lympho-kwik (One Lambda, lnce., Canoga Park, CA) T cell isolation solution for 20 min at 37 C followed by a 5 min centrifugation at 1500 g. The resulting cell pellet was washed twice prior to use. PBMC or freshly isolated T cells were cultured in basic culturing media consisting of RPMI 1 640 (Gibco Laboratories), 10% FCS (Hyclone, Utah), 1 x MEM nonessential amino acids (Sigma), 1 mM sodium pyruvate (Gibco), 2 mM glutamine(Sigma), and 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco) supplemented with 5 Rg/mi PHA (Calbiochem) and 50 @ 2-ME (Sigma).

Of the lymphocyte sized fraction, <2% were positive for 4B4-1 mAb binding (data not shown) when compared to binding observed with an irrevelant-isotype control. By multi-color analysis, <2% of CD3+ (T) cells were found to expressed 4-1 BB.

Isotype-matched irrelevant mAb were used for each fluorescence parameter to correct for non-specific binding of the mabs. By 48 hrs of PHA-stimulation, 18.5-21.5% of purified T cells expressed 4-1 BB on its cell surface. Without PHA-stimulation <2% of T cells express 4-1 BB. On PHA-stimulated T cells, 4-1BB was expressed on both CD4+ and CD8+ cells with ~50% of 4-1 BB+ cells expressing CD4 and 50% expressing CD8.

Binding of 4B4-1 FITC-labeled mAb was almost completely blocked when PHA-stimulated T cells were pre-incubated with μ/ml purified rh4-1 BB protein, but not irrelevant control protein at similar concentration made in a similar manner.

PHA-stimulated T cells were incubated 30 min. on ice with staining media or varying concentrations of either rh4-1 BB protein or irrelevant recombinant protein that was produced in a similar manner as rh4-1 BB. Without washing 2.5 μg of 4B4/FITC-labeled mAb or isotype matched FITC-labeled mAb, a suboptimal concentration, was added and cells were further incubated on ice for 30 min. Cells were washed 3 times with staining media, fixed with 1 % paraformaldehyde, and analyzed by FCM as described above.

The competitive blocking of 4B4-1 /FITC mAb by rh4-1 BB was dependent upon the concentration of rh4-1 BB. Together this data demonstrates that 4B4-1 mAb specifically recognizes the human 4-1 BB on peripheral blood T cells.

Activated T cells co-express 4-1BB+ andCD45RA andCD45RO.

We have previously shown that murine 4-IBB is associate with $p56^{lck}$ by a series of immunoprecipitation studies and peptide mapping study. During the course of these studies we observed that not only did anti-4-1 BB mAb coimmunoprecipitate a 56 kDa protein but it also immunoprecipitated a protein of 200 kDa. Conversely, anti-CD45 mAb co-immunoprecipitated a ~30 kDa protein from ConA stimulated murine thymocytes. This data suggests that 4-1 BB may form a multi-peptide complex with CD45 and $p56^{lck}$ on activated murine T cells. To assess the association of 4-1 BB and CD45 in humans,

PBMCs stimulated with PHA for 48 hrs were analyzed for expression of CD45RA and CD45RO isoforms by multi-color FCM. Sixteen to 19% of cells expressed 4-1 BB, and nearly all (except 1%) expressed CD45RA and nearly all express CD45RO after correcting for non-specific binding of the antibodies.

5

Discussion of Examples of Uses for mAb 4B4-1.

The 4B4-1 mAb specifically stained the T cell line, CEM and peripheral blood T cells following activation, but did not bind resting cells. In this regard, 4-1BB expression in humans is similar to that observed in the mouse. 4B4-1 mAb immunoprecipitates a 10 homodimeric protein of ~35 kDa under reducing conditions and ~70 kDa under non-reducing conditions from 4-1 BB expressing SF-21 cells and activated CEM cells. The molecular weights are similar to the expected values based on amino acid sequence and anticipated glycosylation and similar to molecular weight observed in the mouse. Furthermore, 10 μg of rh4-1 BB protein, but not irrelevant protein, completely block the 15 binding of FITC-labeled 4B4-1 mAb to PHA stimulated T cells. Collectively, these data conclusively show that 4B4-1 mAb is specific for human 4-1 BB.

Although 4B 11 and I G5 mAbs specifically recognize 4-1 BB expressed on SF-21 cells, they do not recognize 4-1 BB expressed on activated T cells. This is most likely due to the mAb having specificity for a cryptic or unique binding site(s) that is not exposed or 20 present on T-cells but is accessible or present on SF-21 cells due to slight differences in glycosylation and processing between human T cells and insect cells (SF-21).

In mice, neither 4-1BB mRNA nor surface expression is detectable on resting splenocytes or unstimulated cloned T cells. But upon activation of T cells by anti-CD3 or antiTCRαβ, 4-1 BB mRNA is detected within 3 hrs of stimulation and is first detectable 25 on the cell surface 2-3 days following stimulation. Maximum surface expression is reached about 6 days following stimulation. As in the mouse, 4-1 BB is not detected on the surface of freshly isolated peripheral blood T cells in man, but is readily detected following PHA-stimulation. Unlike in the mouse, 4-1BB is expressed much more rapidly in humans, reaching a peak expression level within 12-48 hrs. 4-1BB expression begins to

decrease within 72 hrs poststimulation, as do the number of cells expressing 4-1 BB on their cell surface. In both mouse and humans, 4-1 BB is expressed on CD4+ and CDS+ T cell subsets) (19).

4-1 BB is associated with $p56^{lck}$. A 56 kDa protein is detected when $^{32}PO_4$ was transferred from gamma-labeled ATP onto the p56 protein in ConA activated thymocytes that were subjected to immunoprecipitated with anti-4-1 BB mAb, 1AH2. By peptide mapping, this 56 kDa phosphoprotein was identified as $p56^{lck}$. $p56^{lck}$ and 4-1 BB was also be found to co-immunoprecipitate from insect cells (SF-21) and HeLa cells transfected with 4-1BB and $p56^{lck}$. Furthermore, cross-linking of 4-1BB activated $p56^{lck}$. Cysteine residues critical for $p56^{lck}$ -CD4/CD8 complex formation were also critical for $p56^{lck}$ - 4-1BB interaction. In prelimary results, it was noted that anti-4-1BB also immunoprecipitated a protein of ~200 kDa from biotin-surface labeled ConA activated thymocytes. When anti-CD45 mAb was used for immunoprecipitation, a ~30 kDa protein, of similar size to murine 4-1BB, was detected. Other have previously shown that CD45 mediates the dephosphorylation of certain proteins such as $p56^{lck}$ (44). Perhaps 4-1 BB plays a role in bringing CD45 and $p56^{lck}$ together and facilitates the dephosphorylation of $p56^{lck}$ by the CD45 phosphatase.

To further assess the association of 4-1 BB and CD45, PHA-stimulated PBMCs were analyzed by multicolor FCM. Approximately 16-19% of PBMCs cultured in PHA for 48 hrs express 4-1 BB. If all 4-1BB+ cells express CD45RA (Figure 7A) and express CD45RO, the ~17.5% of 4-1BB+ cells must co-express both CD45RA and CD45RO on their cell surface. Of the PHA-stimulated $CD45RA^{hi}RO^{hi}$ cells, approximately 50% express 4-1 BB. This data futher supports the hypothesis that CD45 and 4-1BB share an association. More importantly, it suggests that 4-1 BB may play a role in T cell transition from a naive phenotype ($CD45RA^{hi}RO^{lo}$) to a memory phenotype ($CD45RA^{lo}RO^{hi}$). Picker et al. previously demonstrated through multi-color FCM, that naive T cells undergo a "stepwise, unidirectional progression" from a naive ($CD45RA^{lo}RO^{hi}$) to a memory ($CD45RA^{lo}RO^{hi}$) phenotype through a distinct $CD45RA^{hi}RO^{hi}$ intermediate cell type (21). Peripheral blood few cells that express this intermediate phenotype are detectable. However, in secondary lymphoid tissue, such as tonsil, 2-10% of T cells were found to be

CD45RA^{hi}RO^{hi}. Much is know about the naive and memory T cells, but little is known about the CD45RA^{hi}RO^{hi} in transitional cells. Nor is it known what events occur during this transition phase that result in memory T cell development. Therefore, it will be necessary to assess the role of 4-1 BB in the transition from a naive to that of a memory T cell and the apparent association of 4-1 BB and CD45. In this regard, 4B4-1 mAb will be invaluable.

The mAb 4B4-1 can be used to enhance T-cell cross-linking and therefore induce T-cell activation against certain types of cancer cells (e.g. melanoma). By using the mAb in experiments with various cancer cells in the presence of T-cells dosages and proper formulations of initiating T-cell activation against the cancer cells can be determined. The formulations are tested in animal models with the same type of cancer and the formulations and dosages are refined for testing in humans.

Sinovial T-lymphocytes in patients with rheumatoid arthritis express H4-1BB, but 4-1BB is not expressed in sinovial T-lymphocytes of patients without this disease. This disease involves an undesired immune response against the patient's own tissue. Therefore, blocking the undesired immune response would provide relief for the arthritis sufferer. By injecting the patient with the mAb 4B1-4 or the fusion protein, the binding between H4-1BB and its ligand would be blocked. If the binding of the mAb and H4-1BB did not enhance activation of the immune system, then the mAb 4B1-4 interference with binding would have the desired effect, otherwise the fusion protein would be used for blocking binding. The fusion protein (monomeric) does not stimulate H4-1BB or its ligand but is a good ligand binding blocker because it binds to the H4-1BB ligand thereby preventing H4-1BB from binding and stimulating the ligand.

A similar method of blocking ligand binding would be useful for treating patients with systemic lupus erythematosus. For patients with Type I diabetis - T-cells attack their own insulin producing cells, pancreatic Beta cells. By injecting the mAb or fusion protein this destruction can be blocked.

Peripheral blood T cells in patients with AIDS or certain types of viral flu are expressing H4-1BB, whereas the same cells in normal patients are not expressing H4-1BB.

Therefore, 4-1BB is important in this immune response. The enhancement or blocking of H4-1BB ligand binding or cross-linking will be important in regulating the T-cells in patients with these diseases.

APPENDIX A - REFERENCES

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APPENDIX B
COMMON ABBREVIATIONS

CTL	cytolytic T lymphocyte
HTL	helper T lymphocyte
LGL	large granular lymphocytes
NK	natural killer cells
ConA	concanavalin A
DTT	dithiothreitol
mAb	monoclonal antibody.
4- 1BB	protein expressed on activated T cells
rh) 4-1BB	recombinant human 4-1BB
4-1BB/L	ligand to 4-1BB found on activatedmacropage and mature B cells
4-1BB/AP	fusion protein between 4-1BB and alkaline phosphatase.
SDS	sodium dodecysulfate
SSC	150 mM sodium chloride/15 mM sodium citrate, pH 7.0
TPA	12-0-tetradecanoylphorbol-13-acetate
Th	helper T lymphocytes
IL-2	interleukin 2
IL-3	interleukin 3
rIL-2	recombinant Il-2
CSF-GM	granulocyte/macrophage colony-stimulating factors
cRNA	complementary RNA
ss	single-stranded
ds	double-stranded
TCR	T-cell antigen receptor
PTA	phorbol 12-tetradecanoate 13-acetate
r	recombinant
mu	murine
hu	human
BFU-E	burst forming unit-erythroid, an erythroid progenitor cell
CFU-GEMM	colony forming unit-granulocyte erythroid macrophage

	megakaryocyte, a multipotential progenitor cell
CFU-GM	colony forming unit-granulocyte macrophage, a granulocyte-macrophage progenitor cell
CFU-S	colony forming unit-Spleen, a multipotential stem cell
H-ferritin	the heavy chain subunit form of ferritin
MGF	mast cell growth factor, a c-kit ligand
CSF	colony stimulating factors
G	granulocyte
M	macrophage
Epo	erythropoietin
IL	interleukin
LD	low density
NALDT	non-adherent low density T-lymphocyte depleted
PMSF	phenylmethylsulfonyl fluoride
PBS	phosphate buffered saline
AcNPV	*Autographa californica* nuclear polyhedrosis virus
SDS	sodium dodecyl sulfate
LPS	lipopolysaccharide

The foregoing description has been directed to particular embodiments of the invention in accordance with the requirements of the Patent Statutes for the purposes of illustration and explanation. It will be apparent, however, to those skilled in this art that many modifications and changes will be possible without departure from the scope and spirit of the invention. It is intended that the following claims be interpreted to embrace all such modifications.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Kwon, Byoung Se
Kang, Chang-Yuil

(ii) TITLE OF INVENTION: Monoclonal antibody against human receptor 4-1BB

(iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Barnard, Brown & Michaels
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/122,796
(B) FILING DATE: 16-SEP-1993

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/012,269
(B) FILING DATE: 01-FEB-1993

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/922,996
(B) FILING DATE: 30-JUL-1992

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/267,577
(B) FILING DATE: 07-NOV-1988

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2347 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: murine 4-1BB

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 146..916

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 146..913

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Kwon, Byoung Se
et al.,
(B) TITLE: cDNA sequences of two inducible T-cell genes
(C) JOURNAL: Proc. Natl. Acad. Sci. U.S.A.
(D) VOLUME: 86
(E) ISSUE: March
(F) PAGES: 1963-1967
(G) DATE: 1989
(K) RELEVANT RESIDUES IN SEQ ID NO:1: FROM 1 TO 2347

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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(D) TOPOLOGY: linear

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(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(B) TYPE: nucleic acid
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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
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(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(2) INFORMATION FOR SEQ ID NO:5:

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(C) STRANDEDNESS: single
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(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(2) INFORMATION FOR SEQ ID NO:6:

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(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(B) TYPE: nucleic acid
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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
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(C) INDIVIDUAL ISOLATE: H4-1BB #1
(D) DEVELOPMENTAL STAGE: Differentiated T-cell
(G) CELL TYPE: Lymphocyte

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(D) OTHER INFORMATION: /codon_start= 41
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150                 155                 160                 165

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(2) INFORMATION FOR SEQ ID NO:8:

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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Ala Gly Thr Phe Cys Asp Asn Asn Arg Asn Gln Ile Cys Ser Pro Cys
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Pro Pro Asn Ser Phe Ser Ser Ala Gly Gly Gln Arg Thr Cys Asp Ile
    50                  55                  60

Cys Arg Gln Cys Lys Gly Val Phe Arg Thr Arg Lys Glu Cys Ser Ser
65                  70                  75                  80

Thr Ser Asn Ala Glu Cys Asp Cys Thr Pro Gly Phe His Cys Leu Gly
                85                  90                  95

Ala Gly Cys Ser Met Cys Glu Gln Asp Cys Lys Gln Gly Gln Glu Leu
            100                 105                 110

Thr Lys Lys Gly Cys Lys Asp Cys Cys Phe Gly Thr Phe Asn Asp Gln
        115                 120                 125

Lys Arg Gly Ile Cys Arg Pro Trp Thr Asn Cys Ser Leu Asp Gly Lys
    130                 135                 140

Ser Val Leu Val Asn Gly Thr Lys Glu Arg Asp Val Val Cys Gly Pro
145                 150                 155                 160

Ser Pro Ala Asp Leu Ser Pro Gly Ala Ser Ser Val Thr Pro Pro Ala
                165                 170                 175

Pro Ala Arg Glu Pro Gly His Ser Pro Gln Ile Ile Ser Phe Phe Leu
            180                 185                 190

Ala Leu Thr Ser Thr Ala Leu Leu Phe Leu Leu Phe Phe Leu Thr Leu
        195                 200                 205

Arg Phe Ser Val Val Lys Arg Gly Arg Lys Lys Leu Leu Tyr Ile Phe
    210                 215                 220

Lys Gln Pro Phe Met Arg Pro Val Gln Thr Thr Gln Glu Glu Asp Gly
225                 230                 235                 240

Cys Ser Cys Arg Phe Pro Glu Glu Glu Glu Gly Gly Cys Glu Leu
                245                 250                 255
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR Primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AATAAGCTTT GCTAGTATCA TACCT

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR Primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTAAGATCTC TGCGGAGAGT GTCCTGGCTC

American Type Culture Collection

12301 Parklawn Drive • Rockville, MD 20852 USA • Telephone: (301)231-5520 Telex: 898-055 ATCC NORTH • FAX: 301-770-2587

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3 AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Indiana University Foundation
Attn: Byoung Se Kwon
Indiana University School of Medicine
Department of Microbiology & Immunology
635 Barnhill Drive, MS-255
Indianapolis, IN 46202-5120

Deposited on Behalf of: Indiana University Foundation

Identification Reference by Depositor:	ATCC Designation
Hybridoma that produces 484-1 mAb	HB-11860

The deposit was accompanied by: _ a scientific description _ a proposed taxonomic description indicated above.

The deposit was received March 10, 1995 by this International Depository Authority and has been accepted.

AT YOUR REQUEST:

X We will not inform you of requests for the strain.

The strain will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strain and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strain.

If the culture should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace it with living culture of the same.

The strain will be maintained for a period of at least 30 years after the date of deposit, and for a period of at least five years after the most recent request for a sample. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the culture cited above was tested March 15, 1995. On that date, the culture was viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Annette L. Bade, Director, Patent Depository

Date: March 17, 1995

✓cc: Christopher A. Michaels

What is claimed is:

1. A monoclonal antibody against H4-1BB which preferentially binds to at least a portion of receptor protein H4-1BB.

2. A hybridoma capable of producing a monoclonal antibody of claim 1.

3. A method of using the monoclonal antibody of claim 1 to enhance T-cell proliferation comprising the step of treating T-cells that have expressed receptor protein H4-1BB with said monoclonal antibody.

4. The method of claim 3 further comprising the step of conducting said treatment in the presence of co-stimulatory molecule.

5. A method of using the monoclonal antibody of claim 1 to enhance T-cell activation comprising the step of treating T-cells that have expressed receptor protein H4-1BB with said monoclonal antibody.

6. The method of claim 5 further comprising the step of conducting said treatment in the presence of co-stimulatory molecule.

7. The method of using the monoclonal antibody of claim 1 to inhibit the interaction of H4-1BB and its ligand.

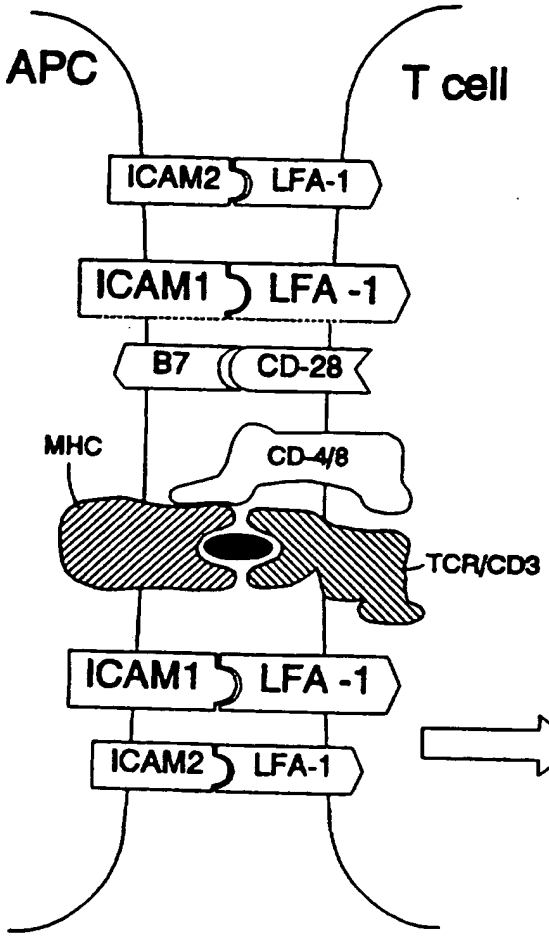
8. The method of using the monoclonal antibody of claim 1 to aid in killing cancer cells by using said monoclonal antibody to activate T-cells against said cancer cells.

9. The method of using the monoclonal antibody of claim 1 to treat autoimmune diseases by using the monoclonal antibody to inhibit the interaction of H4-1BB and its ligand.

10. The method of using the monoclonal antibody of claim 1 to block a patient's immune response during organ transplantation by using the monoclonal antibody to inhibit the interaction of H4-1BB and its ligand.

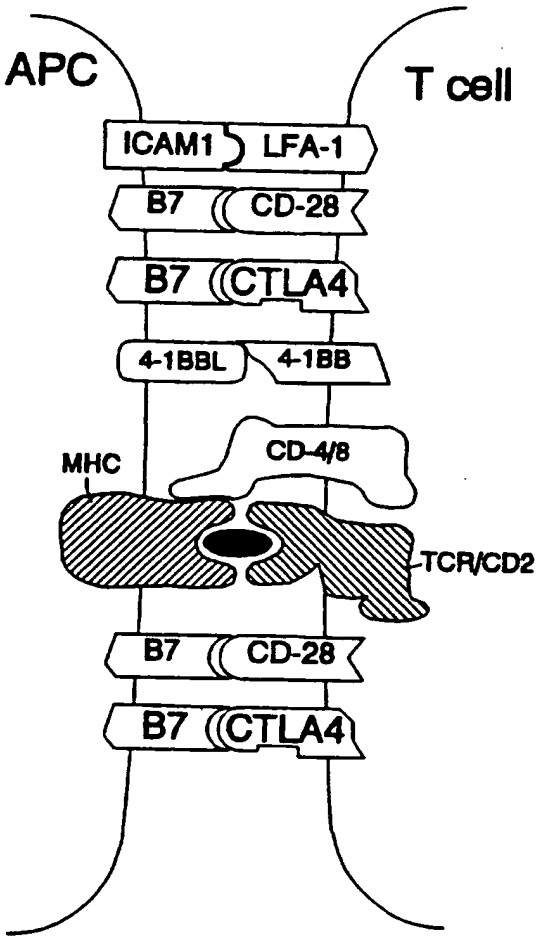
11. An antibody that is immuno reactive with a purified human 4-1BB polypeptide comprising the N-terminal amino acid sequence Leu-Gln-Asp-Pro-Cys-Ser-Asn-Cys-Pro-Ala-Gly-Thr.

12. An antibody of claim 11, wherein said antibody is a monoclonal antibody.



COGNITIVE PHASE
early activation

Fig. 1a



PROLIFERATION
CLONAL EXPANSION
late activation

Fig. 1b

NORMAL T-CELL ACTIVATION PATHWAY

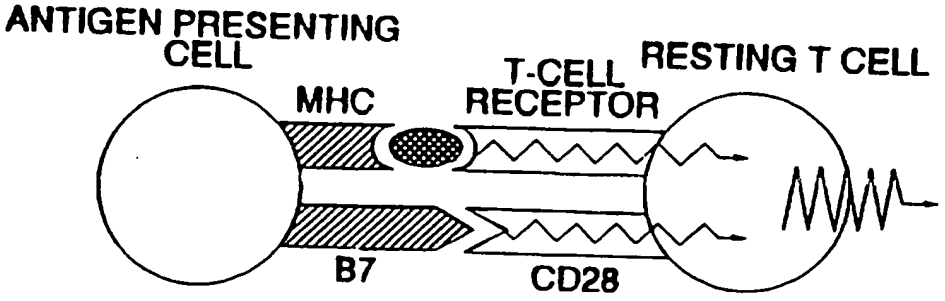


Fig. 2a

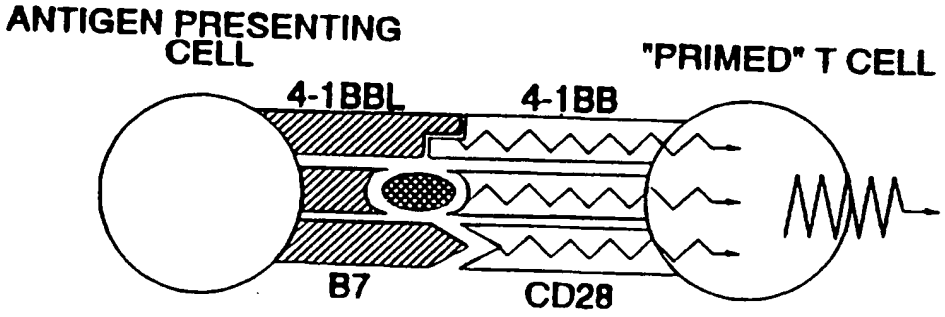


Fig. 2b

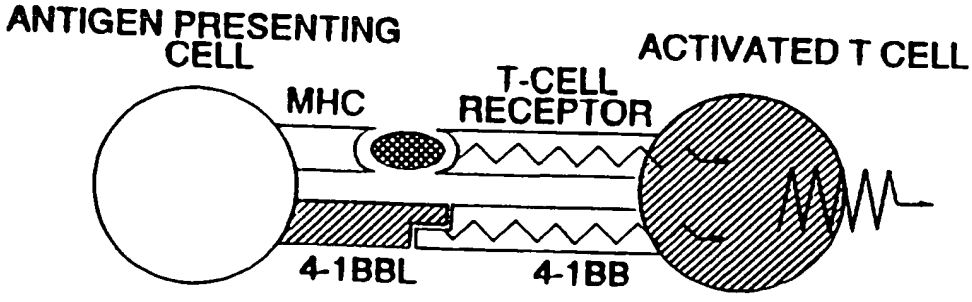


Fig. 2c

BLOCKING STEPS IN T-CELL ACTIVATION PATHWAY

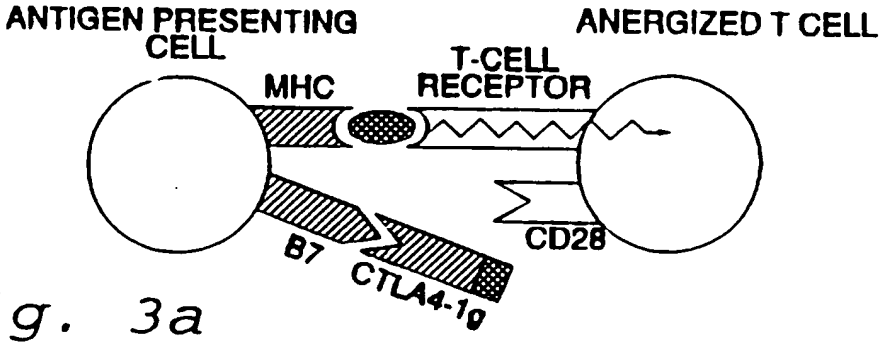


Fig. 3a

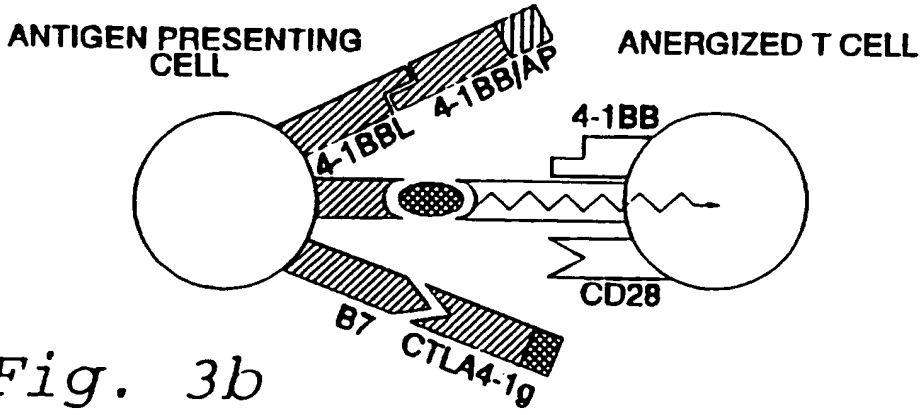


Fig. 3b

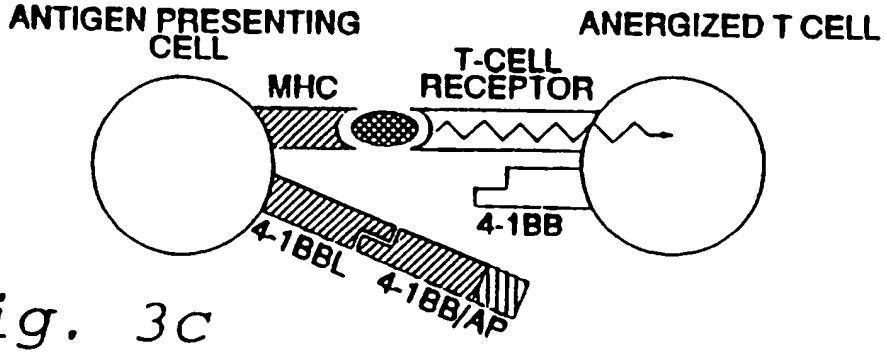


Fig. 3c

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/03965

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 16/00; C12N 5/00; G01N 33/53; A61K 39/395
US CL : 530/387.9, 388.24; 435/7.24, 240.27; 424/145.1, 158.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/387.9, 388.24; 435/7.24, 240.27; 424/145.1, 158.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	ALDERSON, M.R. et al. Molecular and Biological characterization of Human 4-1BB and its ligand. Eur. J. Immunol.. 1994, 24(9), pages 2219-2227.	1-2, 11-12 ---------- 3-10
X -- Y	SCHWARZ, H. et al. ILA, the Human 4-1BB homologue, is inducible in lymphoid and other cell lineages. Blood, 15 Febuary 1995, Vol.85, N.4, pages 1043-1052.	1-2, 11-12 ---------- 3-10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
23 JUNE 1996

Date of mailing of the international search report
02 AUG 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 308-4242

Authorized officer
MINH-TAM B. DAVIS
Telephone No. (703) 305-2008

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/03965

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

DIALOG, WPI, MPSRCH,REGISTRY. Search terms: sequences 2, 8, and the sequence cited in claim 11, antibody, nerve growth factor receptor, tumor necrosis factor, autoimmune disease treatment, cancer treatment, h4-1bb, ILA and lymphocyte activation.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claims 1-6 and claims 11-12 are drawn to : 1) an antibody or a monoclonal antibody against receptor protein H4-1BBB, 2) a hybridoma capable of producing said antibody to enhance T-cells proliferation and activation, and 3) a method of using said monoclonal antibody to enhance T-cell proliferation and activation.
Group II, claims 7,9-10 are drawn to a method of using said monoclonal antibody to treat autoimmune diseases and to block a patient's immune response during organ transplantation.
Group III, claim 8 is drawn to a method of using said monoclonal antibody to aid in killing cancer cells.

The inventions listed as Groups I, II and III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The special technical feature of the Group I invention is an antibody or a monoclonal antibody against H4-1BB, a hybridoma producing said antibody, and a first method of using said monoclonal antibody by treating T-cells with said antibody. The special technical feature of the Group II invention is a second method of using said monoclonal antibody which is to treat autoimmune diseases and to block a patient's immune response during organ transplantation. The special technical feature of the Group III invention is a third method of using said monoclonal antibody which is to aid in killing cancer cells. The techniques and reagents used for producing antibody, hybridoma and treating T-cells of the Group I do not pertain to those used for treating autoimmune diseases, blocking a patient's immune response and treating cancer, and vice-versa. The techniques and reagents used for treating cancer are different from those used for treating autoimmune diseases and for blocking immune response during organ transplantation. Therefore unity of invention is lacking . Treating autoimmune diseases and blocking a patient's immune response during organ transplantation are grouped together in group II because there is no extra burden for the examiner to search both treatments.